

# *BRCA1* epigenetic inactivation predicts sensitivity to platinum-based chemotherapy in breast and ovarian cancer

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Germline mutations in the *BRCA1* or *BRCA2* genes are associated with an increased risk of breast and ovarian cancer development. Both genes are involved in DNA repair, and tumors harboring genetic defects in them are thought to be more sensitive to DNA-damaging agents used in chemotherapy. However, as only a minority of breast and ovarian cancer patients carry *BRCA1* or *BRCA2* mutations, few patients are likely to benefit from these pharmacogenetic biomarkers. Herein, we show that, in cancer cell lines and xenografted tumors, *BRCA1* CpG island promoter hypermethylation-associated silencing also predicts enhanced sensitivity to platinum-derived drugs to the same extent as *BRCA1* mutations. Most importantly, *BRCA1* hypermethylation proves to be a predictor of longer time to relapse and improved overall survival in ovarian cancer patients undergoing chemotherapy with cisplatin.

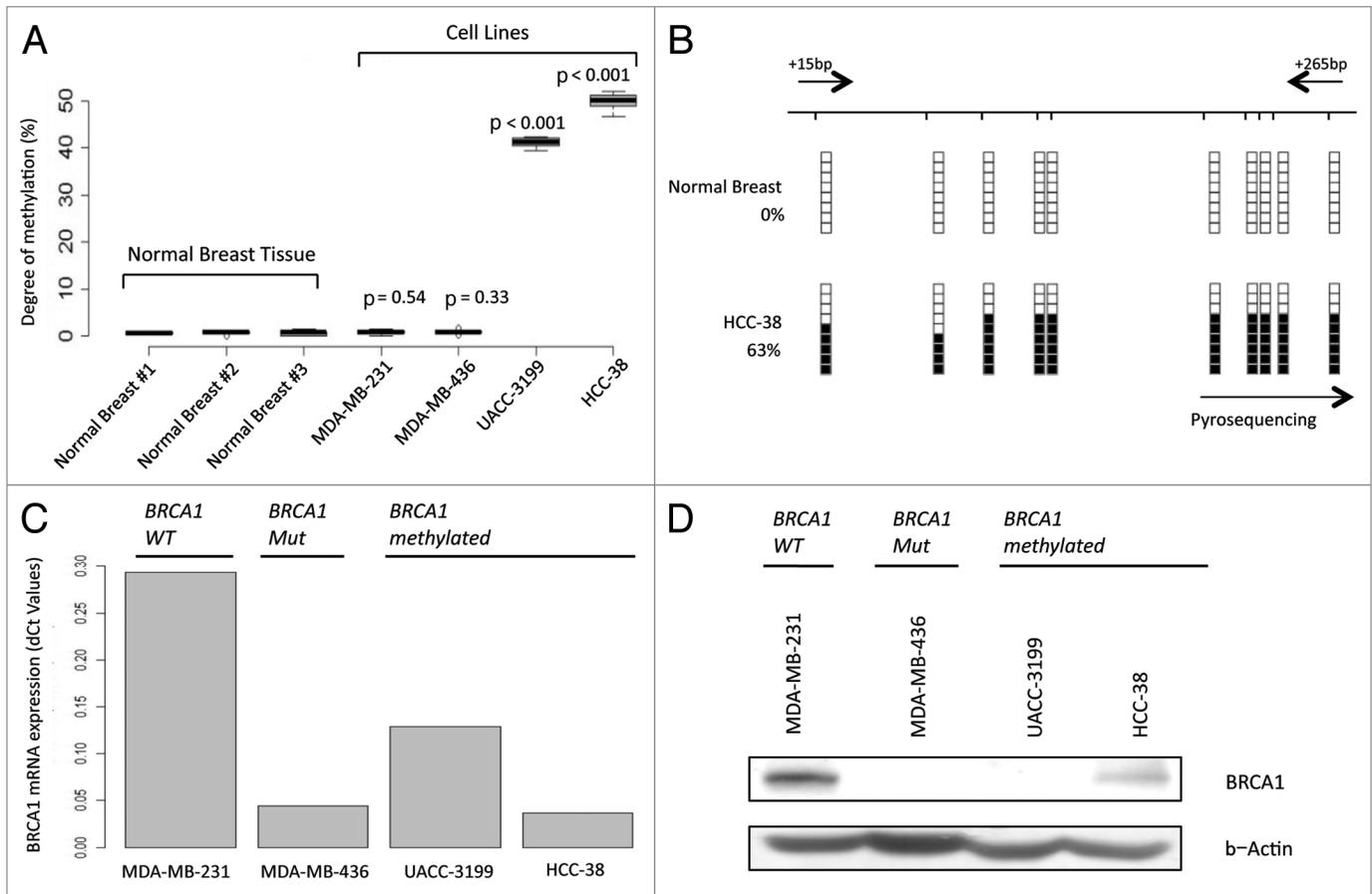
## Introduction

Female *BRCA1* and *BRCA2* mutation carriers have a significantly higher lifetime risk of breast and ovarian cancer.<sup>1</sup> *BRCA1* and *BRCA2* proteins play major roles in DNA double-strand-break repair through homologous recombination,<sup>2</sup> so their deficiencies can impair the capacity of cancer cells to repair DNA cross-links caused by chemotherapy drugs such as platinum-derivatives.<sup>3–7</sup> Ovarian cancer accounts for more deaths than any other tumor of the female reproductive system, so there is great interest in identifying biomarkers for therapy prediction. Two independent studies reported significantly greater primary chemotherapy sensitivity to platinum-based chemotherapy agents in patients with ovarian cancer who were carriers of *BRCA1* and *BRCA2* germline mutations.<sup>5,6</sup> In addition, tumors from carriers of *BRCA1/BRCA2* germline mutations are also sensitive to poly (ADP-ribose) polymerase inhibitors (PARPis) that target the base excision repair pathway.<sup>8–12</sup> However, only a minority of breast and ovarian cancer patients are *BRCA1* and *BRCA2* mutation carriers, so the benefit of these findings might be confined to a small subset of cases. In addition, there might be a link between *BRCA1/BRCA2* defects, platinum sensitivity and response to PARPis in breast and ovarian tumors that is becoming an issue of growing interest.<sup>8–12</sup> Herein, we have approached this matter from a different angle.

In the search for new potential biomarkers of sensitivity differences of human cancer to chemotherapeutic agents, the existence of aberrations in the DNA methylation patterns of cancer cells is turning out to be the most important, particularly those involving hypermethylation of the sequences called CpG islands, which are located in the promoter regions of tumor suppressor genes.<sup>13</sup> One of the most successful discoveries in this area, made by our group<sup>14</sup> and others,<sup>15</sup> and subsequently validated worldwide,<sup>16</sup> is that hypermethylation of the DNA repair enzyme MGMT is associated with a good response to nitrosurea alkylating agents in glioma. For *BRCA1*, there is clear evidence that the *BRCA1* gene can also undergo epigenetic inactivation in sporadic breast tumors<sup>17–22</sup> and ovarian tumors<sup>20,23–25</sup> by the gain of DNA methylation in its promoter-associated CpG island. That this aberration produces a tumor with a *BRCA1* phenotype was further demonstrated by showing that it gives rise to the same pattern of gene expression as seen in inherited *BRCA1* mutations.<sup>26</sup> Strikingly, we and others have recently found that *BRCA1* CpG island hypermethylation also predicts sensitivity to PARPis.<sup>27,28</sup>

We examined whether the enhanced platinum-based sensitivity observed in *BRCA1/BRCA2* familial tumors is also present in sporadic *BRCA1* hypermethylated tumors.

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**Figure 1.** *BRCA1* promoter CpG island hypermethylation is associated with transcriptional silencing. (A) Pyrosequencing analysis of *BRCA1* CpG island demonstrates hypermethylation in UACC-3199 and HCC-38 cancer cells. (B) Bisulfite genomic sequencing of eight individual clones in the *BRCA1* promoter CpG island: examples of a normal breast and the breast cancer cell line HCC-38 are shown. Presence of a methylated or unmethylated cytosine is indicated by a black or white square, respectively. Black arrows indicate the position of the bisulfite genomic sequencing primers. (C) Real-time PCR expression of the *BRCA1* transcript. (D) *BRCA1* expression was also determined by western blot and the  $\beta$ -actin protein was used as a loading control. The UACC3199 and HCC-38 breast cancer cells show a hypermethylated CpG island in association with the downregulation of the *BRCA1* protein. MDA-MB-231 (wild-type) and MDA-MB-436 (mutant) are shown as positive and negative controls for *BRCA1* expression.

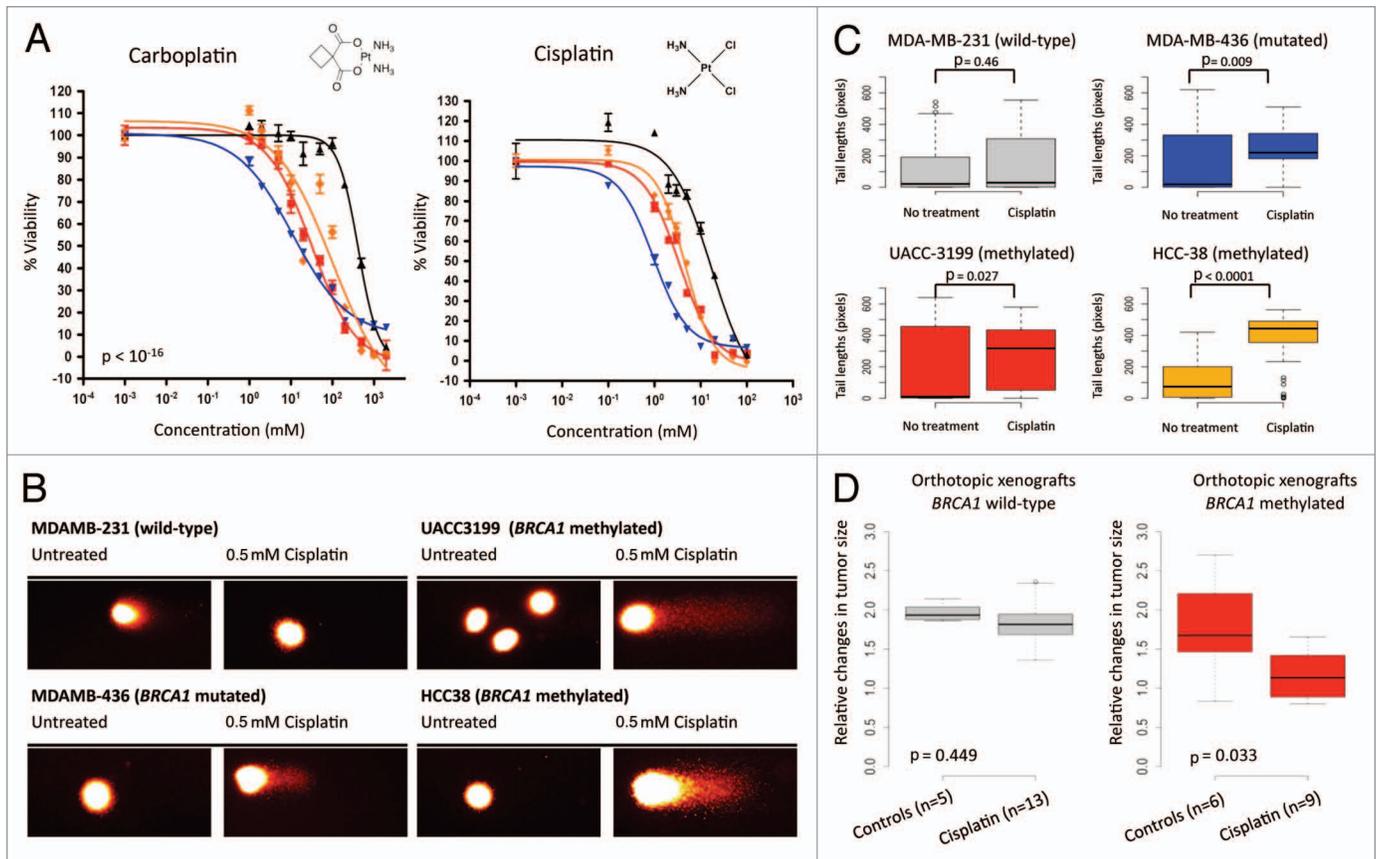
## Results and Discussion

*BRCA1* and *BRCA2* are candidate genes for hypermethylation-associated inactivation in human cancer because a 5'-CpG island is located around the corresponding transcription start sites. To analyze the methylation status of the promoter-associated CpG islands, we screened 15 human cancer cell lines from breast (HCC-1143, MDA-MB-468, MDA-MB-468-PT, MDA-MB-468LN, MCF7, SK-BR-3, T47D, Hs578T, UACC3199, MDA-MB-231 and MDA-MB-436) and ovarian (SK-OV-3, IGR-OV1, OVCAR-3 and OVCAR-5) tumor types, using bisulfite genomic sequencing, methylation-specific PCR and pyrosequencing. *BRCA2* promoter CpG island methylation was not found in any of the cases, but the breast cancer cell lines UACC3199 and HCC-38 exhibited *BRCA1* CpG island promoter hypermethylation (Fig. 1). All normal breast tissues analyzed were completely unmethylated at the *BRCA1* promoter CpG island (Fig. 1).

Having noted *BRCA1* promoter hypermethylation in the aforementioned cancer cell lines, we assessed the association

between this epigenetic aberration and the putative transcriptional inactivation of the *BRCA1* gene at the RNA and protein levels. The cancer cell lines UACC3199 and HCC-38 hypermethylated at the *BRCA1* CpG island had minimal expression of the *BRCA1* RNA transcript, as determined by quantitative RT-PCR (Fig. 1), and *BRCA1* protein, as determined by western blot (Anti-*BRCA1* Ab-1, Calbiotech, Clone# MS110) (Fig. 1). The *BRCA1* mutant breast cancer cell line MDA-MB-436 cell, which carries a genetic deletion, was used as a control for the lack of expression of the *BRCA1* transcript and protein (Fig. 1). In contrast, the *BRCA1* unmethylated and non-mutant MDA-MB-231 cell line expressed the *BRCA1* transcript and protein (Fig. 1).

An increasing number of reports suggest that tumors with genetic defects in *BRCA1* are more sensitive to growth inhibition and chromosomal damage upon platinum-based chemotherapy. This makes it extremely interesting to know, for clinical translational purposes, whether cancer cells with *BRCA1* methylation-associated silencing also possess these functional features. First, we studied the antiproliferation effects of cisplatin and



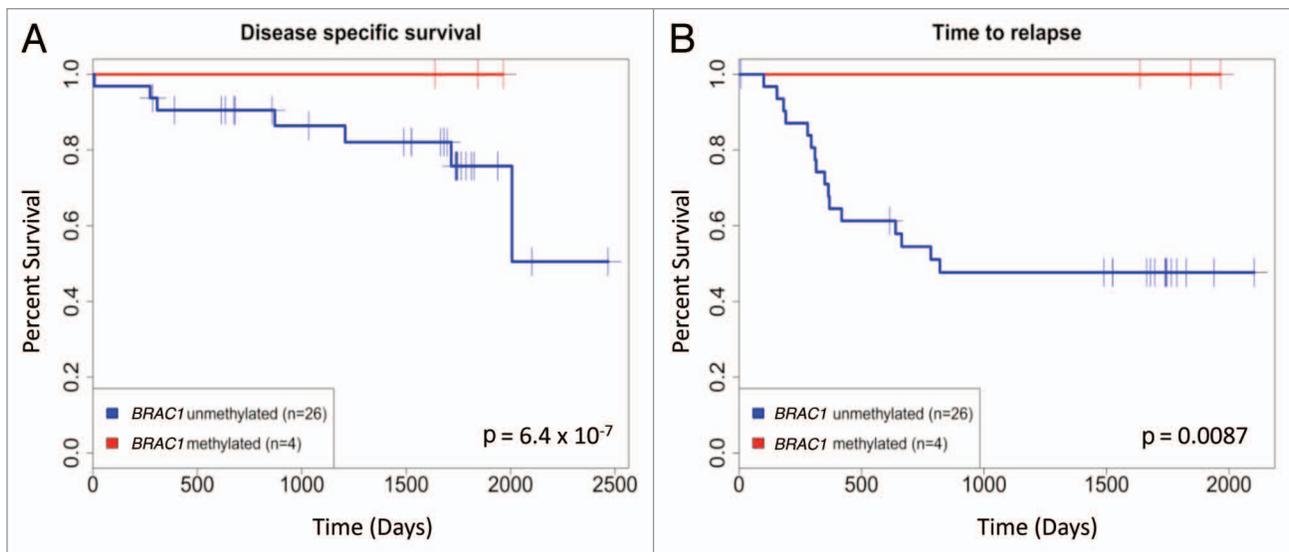
**Figure 2.** *BRCA1* epigenetic inactivation predicts sensitivity to platinum-based chemotherapy. (A) Cell viability assessed by the MTT assays demonstrates that methylated (UACC3199 and HCC-38) and mutant (MDA-MB-436) *BRCA1* cells both exhibit enhanced sensitivity to cisplatin and carboplatin in comparison with wild type and unmethylated MDA-MB-231 breast cancer cells. The corresponding  $IC_{50}$  values are shown. (B) Representative comet assays show DNA damage upon cisplatin use in the *BRCA1* methylated or mutated cell lines. (C) Quantification of the obtained values from the comet assay. *BRCA1*-hypermethylated cells are not able to repair DNA damage when cisplatin is used. The values of comet assays shown in box-plots demonstrate that both methylated and mutant *BRCA1* cells experience permanent DNA damage when cisplatin is used that it is not observed in *BRCA1* wild type or unmethylated cells (MDA-MB-231). (D) Relative changes in tumor size of UACC3199 (*BRCA1* hypermethylated) and MDA-MB-231 (*BRCA1* unmethylated) cancer cells xenografted in nude mice upon cisplatin use. Values shown at 28 d after the start of the chemotherapy treatment.

carboplatin in the four described cancer cell lines with different *BRCA1* genetic/epigenetic status using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. *BRCA1* hypermethylation (UACC3199 and HCC-38 cells) conferred the same degree of sensitivity to the two platin compounds as did the *BRCA1* mutation (Fig. 2), while the unmethylated and non-mutated cell line (MDA-MB-231) was significantly more resistant (Fig. 2). Related to the formation of double-strand breaks in the DNA upon the use of the platin-derivatives, *BRCA1*-deficient cells (hypermethylated or mutated) experienced equally massive DNA damage, as assessed by the comet assay, when treated with cisplatin or carboplatin (Fig. 2). This was not observed in *BRCA1*-proficient cells (Fig. 2). It is interesting to note that *BRCA1* unmethylated and non-mutated cells express increasing amounts of *BRCA1* when platin is used, enabling the effective repair of induced DNA lesions, but *BRCA1* hypermethylated cells are unable to experience this reactive change (Fig. 2).

We transferred our experiments from the in vitro assays described above to an in vivo setting in a mouse model. The antitumor activity of cisplatin was evaluated with respect to

*BRCA1* epigenetic status using UACC3199 (*BRCA1* hypermethylated) and MDA-MB-231 (*BRCA1* unmethylated) cancer cells xenografted in nude mice. Upon subcutaneous administration of cisplatin, significant tumor growth inhibition over time was observed in the *BRCA1* hypermethylated xenografts ( $p = 0.025$ ), but not in unmethylated cells ( $p = 0.443$ ). The mice were sacrificed 30 d after the start of the treatment and the tumor size of the xenograft was measured. *BRCA1* hypermethylated cells had significantly smaller tumors than the xenografted unmethylated cells ( $p = 0.033$ ) (Fig. 2).

Given the aforementioned in vitro and in vivo findings that human cancer cells with *BRCA1*-methylation-associated silencing are very sensitive to platin derivatives, we wondered whether the same could be observed in clinical samples. In the clinical context, cisplatin is a chemotherapy drug widely used in the treatment of ovarian cancer, a tumor type in which a significant rate of *BRCA1* CpG island hypermethylation has been described.<sup>17-22</sup> We therefore assessed whether the presence of *BRCA1* promoter CpG island hypermethylation, detected by pyrosequencing, was a predictive marker of response to cisplatin in ovarian cancer



**Figure 3.** *BRCA1* hypermethylation proves to be a predictor of good response to chemotherapy with cisplatin in ovarian cancer patients. (A) *BRCA1* hypermethylation in patients with ovarian cancer is associated with longer time to relapse. (B) *BRCA1* hypermethylation in patients with ovarian cancer is associated with improved disease-specific survival.

patients treated with this drug. The study of a well characterized clinical cohort of serous epithelial ovarian tumors [FIGO stages: I (n = 7), II (n = 3), III (n = 18) and IV (n = 2)], all of which were treated with cisplatin, showed that *BRCA1* methylation was observed in 13% (4 of 30) of the cases. The *BRCA1* hypermethylated ovarian tumors corresponded to FIGO stages I (n = 2) and II (n = 2). Most importantly, *BRCA1* epigenetic inactivation was associated with a significantly longer time to relapse (Cox regression, log-rank,  $p = 6.40E-007$ ) and improved overall survival (Cox regression, log-rank,  $p = 0.009$ ) (Fig. 3). Thus, the clinical data resemble the aforementioned cell culture and xenograft results that suggest an increased chemosensitivity of *BRCA1* hypermethylated tumors to platinum-derived drugs.

One of the “holy grails” of current medical oncology is personalized cancer treatment. The oncologist would like to have information available that pinpoints a particular molecular Achilles’ heel in a given patient that indicates the usefulness of a particular drug. To date, this approach has been most successful for treating hematological malignancies, but progress with solid tumors, such as breast, colon and lung tumors has also been made. A number of studies in ovarian tumors<sup>5,6</sup> support the hypothesis that

inherited genetic defects in *BRCA1/BRCA2* render these neoplasms more sensitive to platinum-based regimens. Herein, using the *BRCA1* epigenetic defect, we have broadened these observations to include sporadic tumors, which make up the vast majority of cases attended by medical practitioners. Our results support the inclusion of *BRCA1* promoter CpG island hypermethylation in biomarker panels assessing the clinical efficacy of platinum-based chemotherapy.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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